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REVIEW

Potential Use of T Cell Receptor Genes to Modify Hematopoietic Stem Cells for the Gene Therapy of Cancer

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The purpose of this review is to illustrate some of the technical and biological hurdles that need to be addressed when developing new gene therapy based clinical trials. Gene transfer approaches can be used to "mark" cells to monitor their persistence in vivo in patients, to protect cells from toxic chemotherapeutic agents, correct a genetic defect within the target cell, or to confer a novel function on the target cell. Selection of the most suitable vector for gene transfer depends upon a number of factors such as the target cell itself and whether gene expression needs to be sustained or transient. The TCR gene transfer approach described here represents one innovative strategy being pursued as a potential therapy for metastatic melanoma. Tumor reactive T cells can be isolated from the tumor infiltrating lymphocytes (TIL) of melanoma patients. A retroviral vector has been constructed containing the T cell receptor (TCR) α and β chain genes from a MART-1$_{(27-35)}$-specific T cell clone (TIL 5). Jurkat cells transduced with this virus specifically release cytokine in response to MART-1$_{(27-35)}$ peptide pulsed T2 cells, showing that the virus can mediate expression of a functional TCR. HLA-A2 transgenic mice are being used to examine whether transduced bone marrow progenitor cells will differentiate *in vivo* into mature CD8$^+$ T cells expressing the MART-1$_{(27-35)}$-specific TCR. Expression of the human TCR α and β chain genes has been detected by RT-PCR in the peripheral blood of HLA-A2 transgenic mice reconstituted with transduced mouse bone marrow. Expression of the TIL 5 TCR genes in the peripheral blood of these mice was maintained for greater than 40 weeks after bone marrow reconstitution. TIL 5 TCR gene expression was also maintained following transfer of bone marrow from mice previously reconstituted with transduced bone marrow to secondary mouse recipients, suggesting that a pluripotent progenitor or lymphocyte progenitor cell has been transduced. (Pathology Oncology Research Vol 5, No 1, 3–15, 1999)

Keywords: adoptive immunotherapy, gene therapy, stem cell, T cell receptor, tumor antigen

Introduction

The ability to transfer genes from one cell to another has opened the possibility of new treatment strategies designed to correct congenital genetic defects, block the function of deleterious genes, and confer new functions to cells. To date, over 200 gene therapy clinical protocols have been approved worldwide for diseases ranging from HIV to cancer *(Table 1)*. The first gene therapy trial conducted in humans was a gene marking study using adoptively transferred tumor infiltrating lymphocytes (TIL), transduced *ex vivo* with a retrovirus containing the neomycin resistance gene.[1] Gene modified cells were detected in the peripheral blood of these patients as long as 90 days after the transfer. The vast majority of the trials have used potentially therapeutic genes rather than marker genes. The results from studies have clearly demonstrated that genes can be safely introduced into human cells and long term stable expression is possible.

The key to a successful gene therapy protocol is to identify one or a few genes whose transfer can have a significant impact on human disease. A list of therapeutic genes which are currently being tested, their target cells, and the diseases being treated are listed in *Table 2*. Gene

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Table 1. **Gene Therapy Protocols Approved for Clinical Trial**

Disease	*Number of Protocols*[1] *Marker Genes*	*Therapeutic Genes*
Cancer	24	120
Congenital Diseases	0	37
HIV	2	16
Transplantation	6	1
Autoimmunity	0	1
Other	0	5
Total	32	180

[1]Compiled from Human gene marker/therapy clinical protocols, Human Gene Therapy, 8:1499, 1997

therapy can impact the modified cells in several important ways. A normal copy of a defective gene can be inserted which can correct the deficiency, examples of which include the adenosine deaminase gene in SCID patients,[2] the dystrofin gene in muscular dystrophy patients,[3] and the CFTR gene in cystic fibrosis patients.[4] Other genes can be inserted into cells in order to reverse the normal function of a deleterious gene resulting in a change in the phenotype of the cells. Examples of genes which alter the function of a deleterious gene include an anti-sense p53 gene which can inhibit the growth of cancer cells[5], an anti-sense TAR gene in HIV and mutant rev genes which block viral replication in HIV infected cells.[6] Another group of genes can confer novel functions to the cells of interest, such as cytokine genes (IL-2, GM-CSF, TNF and γ-interferon) whose expression can enhance the immunogenicity of the modified tumor cells,[7] the MDR gene which can render hematopoietic stem cells and their progeny resistant to high dose chemotherapy,[8] and antigen receptor genes which can redirect the specificity of lymphocytes (Clay et al., submitted).[9,10]

The development of vector systems that efficiently transfer and express these gene(s) into the appropriate cell type is crucial to the success of any gene therapy protocol. Several different vectors have been tested for use in humans. The major advantages and disadvantages of each vector are listed in *Table 3*. Most gene therapy protocols have used retroviral vectors. The principle reason is that long term stable transgene expression is necessary to effectively treat most diseases. Long term gene expression has frequently been achieved using retroviral vectors.[11] There are reports of stable integration and expression of transferred genes using Adeno-associated virus vectors and naked DNA, but neither has yet been tested extensively in human clinical trials.[12,13,14] However, there are protocols which require only transient expression of the introduced gene and these protocols often use Adenovirus and Pox virus vectors. For example, tumor associated antigen genes, cytokine genes, or co-stimulatory molecule genes need only to be expressed long enough to elicit an anti-tumor immune response.

There are several other considerations when selecting a vector for gene delivery. A major advantage of retroviral vectors over the other viral vectors is the ability to generate stable packaging cells lines which produce virus of consistent titer that is free of replication competent virus *(Table 3)*. As a result, it is relatively easy to produce large quantities of clinical grade virus for patient treatment. One disadvantage to retroviral vectors is their cell cycle dependence for integration. Consequently, non-dividing cells

Table 2. **Genes for Potential Gene Therapy**

Disease	*Gene*	*Target Cell*	*References*
SCID	Adenosine Deaminase	T Lymphocytes	33,34
Cancer	TNF, IL-2,	T Lymphocytes	35,36
Cancer	IL-2	Tumor Cells	36-44
Cancer	TNF, IL-4, Il-7, IFN-?, GM-CSF	Tumor Cells	45-50
Cancer	IL-2, IL-4, IL-12	Fibroblasts	51-53
Cancer	MDR	Hematopoietic Stem Cells	54-57
Cancer	anti-sense to IGF , c-myc, c-fos	Tumor Cells	58-60
Cancer	p53	Tumor Cells	61,62
Cancer	CEA	Dendritic Cells	63
Cancer	Thymidine kinase	Tumor Cells	64,65
AIDS	rev	Hematopoietic Stem Cells	66-68
AIDS	ribozyme, anti-sense-TAR	T Lymphocytes	69-70
Cystic Fibrosis	CFTR	Lung	71-79
Gaucher Syndrome	Glucocerebrosidase	Hematopoietic Stem Cells	80,81
Fanconi Anemia	Fanconi anemia complementation group C	Hematopoietic Stem Cells	82
X-linked CGD	gp91 phox	Hematopoietic Stem Cells	83
Hunter Syndrome	Iduronate-2-Sulfatase	Lymphocytes	84

Table 3. **Vectors for Gene Transfer**

	Retrovirus	*Adenovirus*	*AAV*	*Vaccinia*	*Fowlpox*	*Naked DNA*
Efficiency	Moderate	High	Moderate	High	High	Low
Stable Gene Transfer	Yes	No	Yes	No	No	No
Cell Cycle Dependence	Yes	No	Yes	No	No	No
Stable Producer Lines	Yes1	No	No	No	No	N/A
Titer	Low	High	High	High	High	N/A
Expression of Introduced Genes	Variable	Variable	Variable	High	High	Variable
Expression of Viral Proteins	No	Yes	No	Yes	Yes	No[2]
Risk to Patients	Low	Low	Low	Low	Low	Low

[1]Although virus from stable producer lines is generally used, transient retrovirus production is also possible.
[2]Expression of antibiotic resistance genes is possible for some plasmid vectors.

such as muscle or brain cells are poorly infected with retroviral vectors. For these types of cells, replication incompetent Adenovirus vectors and Adeno-associated virus (AAV) vectors are often chosen. Another important consideration is that Adenovirus and Pox virus vectors express several viral proteins which render them highly immunogenic. Cells infected with these viruses are rapidly eliminated by the host immune system.[15] The immunogenicity of Adenovirus and Pox viruses may make it impossible for patients to be treated more than once with virus or with cells infected with virus *ex vivo*. In contrast, the viral encoded proteins of retroviral vectors and AAV vectors have been deleted (see below) reducing their immunogenicity. Retrovirally transduced cells should not be recognized and eliminated by the host immune system. After considering all of the factors which influence gene transfer, expression, and safety, the vector of choice for most gene therapy protocols remains the retrovirus.

Most retroviral vectors are modified from the Moloney Murine Leukemia Virus (MMLV). Wild type MMLV contains all of the elements necessary for viral replication *(Figure 1 a)*. These include 5' and 3' LTRs which are necessary for integration and contain the promoter elements for transcription of the retroviral genome, the ψ site which is required for packaging the viral genome, and the RNA splicing signals (SD, splice donor and SA, splice acceptor). Other essential components of the virus include the *gag* gene which encodes the structural framework of the virus core, the *pol* gene which encodes reverse transcriptase for viral replication, and the *env* gene whose product mediates viral attachment to target cells which is necessary for efficient virus infection. Two significant modifications have been made to the MMLV backbone for use as a gene delivery vector *(Figure 1 b)*. First, the *gag*, *pol* and *env* genes have been deleted to prevent the production of replication competent virus and to accomodate the insertion of foreign genes. Second, the ATG start codon for the *gag* gene was mutated to prevent translation initiation upstream of the gene(s) of interest. Two of the most common vector designs are illustrated by the LXSN[16] and SAMEN backbones.[17] *(Figure 1 c, d)*. Both vectors contain multiple cloning sites (MCS) for inserting foreign

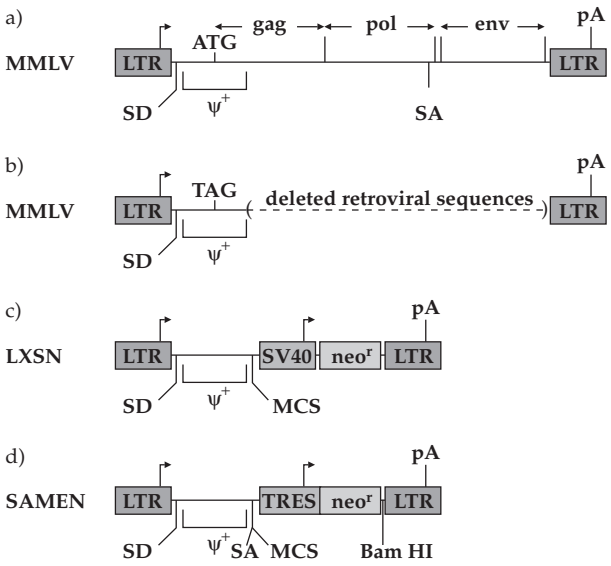

Figure 1. *Recombinant Retroviral Vectors. Wild type Moloney Murine Leukemia Virus (MMLV) (a) contains 5' and 3' LTRs, the ψ packaging signal, splice donor (SD) and splice acceptor (SA) sites, the gag, pol, and env genes. Transcription is initiated in the 5' LTR (denoted by arrow) and polyadenylation occurs from sites located in the 3' LTR (pA). The MMLV genome was modified to create a replication incompetent virus (b). The gag, pol, and env genes were deleted from the MMLV and the ATG translation initiation codon for the gag gene was mutated to TAG to prevent translation of sequences upstream from the foreign gene. The LXSN retroviral vector (c) has multiple cloning sites (MCS) containing unique Eco RI, Hpa I, Xho I, and Bam HI restriction sites for insertion of foreign genes followed by the neo^r gene for G418 selection transcribed by an SV40 promoter. The SAMEN retroviral vector (d) has MCS containing unique Not I, Sna BI, Sal I, Cla I, and Xho I upstream and a unique Bam HI site downstream of the neo^r gene for insertion of foreign genes. An IRES sequence was inserted to facilitate translation of the neo^r gene.*

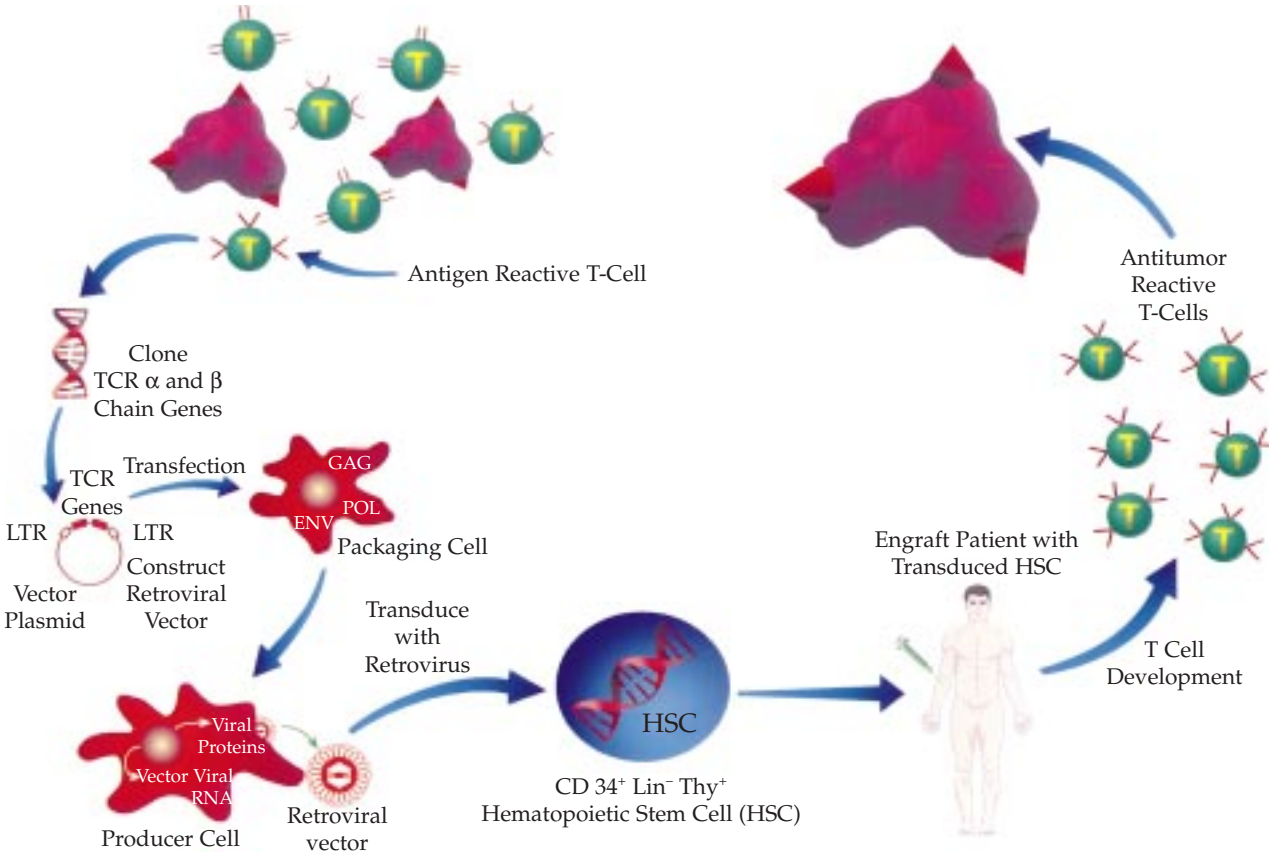

Figure 2. *Scheme for reconstituting cancer patients with TCR transduced hematopoietic stem cells. Tumor reactive T cell clones were isolated and expanded from the TIL or PBMC of patients with metastatic cancer. TCR genes which mediated recognition of TAA were identified and cloned. Retroviral vectors were constructed which can transfer TCR α and β chain genes to alternate effectors. Hematopoietic stem cells were transduced with TCR α and β chain genes and could potentially be used to reconstitute patients.*

genes and the neomycin resistance gene (neo^r) to permit selection of transduced cells with G418. The SA sequence has been inserted just upstream of the MCS in the SAMEN vector resulting in enhanced viral titer. These vectors can accomodate up to 6 kb of foreign DNA and are unable to produce wild type virus making them suitable for most clinical and biological studies.

Since the genes necessary for viral replication are deleted in vectors like LXSN and SAMEN, production of recombinant virus requires trans-acting factors which can be provided by helper viruses or packaging cell lines. Packaging cell lines such as GP&E 86,[18] PG13,[19] and PA317[20] are NIH 3T3 cells stably transfected with the *gag*, *pol*, and *env* genes. The major difference between the various packaging cell lines is the host specificity of the virus which is conferred by the *env* gene. GP&E 86 contains the MMLV envelope and produces an ecotropic virus which infects only rodent cells, PA317 contains the amphotropic murine leukaemia (AM-MLV) virus envelope and produces an amphotropic virus which infects other species including human cells, and PG13 contains the GALV (gibbon ape leukemia virus) envelope and produces a virus which infects only primate cells. The expression of the receptors which bind each of these envelopes is variable from cell to cell. Therefore, it is necessary to test the different packaging cell lines to determine which envelope is best suited for transducing a given cell type.

The Surgery Branch, NCI has initiated several gene therapy trials with the goal of manipulating the host immune system to eliminate or halt the progression of tumors in patients with metastatic cancer. Our previous experiences using tumor infiltrating lymphocytes (TIL) indicated that adoptively transferred T cells had the capacity to traffic to tumor lesions[21] and to mediate regression of established metastatic tumors in animals[22,23,24,25,26] and in cancer patients.[27] These properties and the ability to manipulate the cells *ex vivo* indicated that T cells and T cell progenitors would make excellent targets for gene therapy.

One gene transfer approach currently under development is designed to transfer the reactivity and specificity of a melanoma reactive CTL clone to hematopoietic stem cells. The approach we have developed for transferring the reactivity of T cells to hematopoietic stem cells is shown in *Figure 2*. T cell clones were isolated which specifically rec-

ognize tumor associated antigens (TAA). The T cell receptor (TCR) genes were identified and cloned from these tumor reactive T cell clones. Retroviral vectors containing the TCR α and β chain cDNAs were constructed and high titer producer cell lines were isolated. Hematopoietic stem cells were transduced with the retrovirus containing the TCR genes and could potentially be used to reconstitute patients. Our hypothesis is that, following engraftment, these gene modified hematopoietic stem cells would differentiate into mature T cells bearing the introduced TCR. T cells derived from the gene modified hematopoietic stem cells would express the transferred TCR and would have the reactivity and specificity of the original T cell clone. If this approach were to be successful, TCR genes from tumor reactive T cell clones would represent "off the shelf" reagents that could be used to treat large numbers of patients. This approach is being modeled *in vivo* using HLA-A2 transgenic mice. Mouse bone marrow transduced with TCR genes is being used to engraft lethally irradiated mouse recipients.

The TAA MART-1 is expressed by the majority of human melanoma tumors, making it an excellent potential target for therapeutic strategies utilizing TCR gene transfer. A single HLA-A2-restricted peptide epitope has been identified in the MART-1 protein (amino acids 27–35: MART-$1_{(27-35)}$). HLA-A2 is present in approximately 50% of Caucasian melanoma patients. Therefore, a TCR gene transfer approach using a TCR that recognizes this particular MHC-peptide complex could potentially treat 50% of Caucasian melanoma patients. The TCR used for the studies described here was isolated from a tumor infiltrating lymphocyte culture derived from a melanoma tumor from patient 501. A MART-1 specific T cell clone (TIL 5) was isolated from the TIL 501 culture and the TCR α and β chain cDNAs from this clone were inserted into a retroviral vector for gene transfer.

Materials and Methods

Mice

Breeding pairs of HLA-A2/K^b transgenic mice were kindly supplied by Dr. Linda Sherman (Scripps, La Jolla, CA). C3H and C56Bl/6 mice were obtained from the Frederick Cancer Research Center (Frederick, MD). All mice used in this study were bred and maintained in the NIH B2B animal facility in accordance with animal care and use protocols approved by the National Institutes of Health.

Cells

TIL cultures used in this study were established in the Surgery Branch, NCI from patients with metastatic melanoma. TIL1200 and TIL1235 were $CD8^+$ TIL cultures that recognize melanoma antigens gp100 and MART-1 respectively in the context of HLA-A2.[28] TIL were maintained at a density between $5x10^5$ and $2x10^6$ cells/ml in AIM V medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 5% pooled human AB serum (Sigma, St. Louis, MO) and Pen-Strep-L-glutamine (GIBCO/BRL). NIH3T3, HCT116, Jurkat, T2, and COS cells were maintained in complete medium (RPMI-10) which consisted of RPMI 1640 medium (Gibco/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL), Pen-Strep-L-glutamine (GIBCO/BRL) and 6000 IU/ml recombinant human interleukin 2 (rhIL-2; Chiron, Emeryville, CA). Clone 22 cells (TIL 5 TCR transfected Jurkat cells) were maintained in RPMI-10 medium supplemented with 1 mg/ml G418. GP&E 86 (ecotropic), PA317 (amphotropic), and PG13 (Gibbon Ape) retroviral packaging cell lines were maintained in RPMI-10 medium.

Transient Transfections

COS-7 cells were transiently transfected using the DEAE-dextran method as described.[29] Briefly, 10^6 COS-7 cells in 55 cm^2 dishes were incubated at 37°C for 4 hours in 9 ml DMEM (Biofluids, Rockville, MD) containing 0.5 mg/ml DEAE-dextran (Sigma, St. Louis, MO), 0.1 mM Chloroquine (Sigma), and 10 µg of pcDNA3 (Invitrogen, Carlsbad, CA) containing cloned melanoma associated antigen (MAA) and/or HLA cDNA. Medium was removed and cells were incubated for 2 minutes with 9 mls of 10% DMSO (Sigma) in Hanks Basic Salt Solution (HBSS, Biofluids). Cells were washed twice with HBSS and cultured for 2 days in DMEM medium supplemented with 10% fetal bovine serum (GIBCO/BRL) and Pen-Strep-L-glutamine (GIBCO/BRL).

Cytokine Release Assays

The ability of TIL 5 TCR transduced Jurkat cells to recognize antigen was measured in an IL-2 release assay as described.[30] Briefly, T2 cells ($1x10^6$ cells/ml) were incubated with 2 µg/ml MART-$1_{(27-35)}$ or gp$100_{(209-217)}$ peptides for 2 hours at 37ºC. Cells were washed and 1 x 10^5 peptide pulsed T2 cells were co-cultured with 1 x 10^5 Jurkat responder cells in 200 µl of RPMI-10 containing 10 ng/ml phorbol myristate acetate (PMA, Sigma) in 96-well plates for 24 hours at 37°C. As a positive control, responder cells were incubated with immobilized anti-CD3 monoclonal antibody (OKT3; Ortho, Raritan, NJ). The amount of IL-2 released in culture supernatants was assessed by IL-2 ELISA (R&D Systems, Minneapolis, MN). Recognition of murine MART-1 (m-MART-1) by human MART-1 (h-MART-1) reactive TIL1235 was measured in GM-CSF release assays as described above with the following modifications. COS-7 cell transiently transfected with MAA and HLA-A2 cDNAs were used as

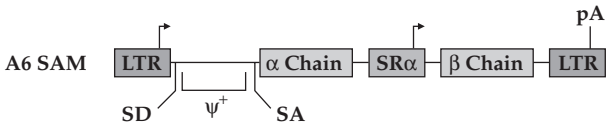

Figure 3. *Structure of A6 SAM retroviral vector. Full length cDNA encoding the α and β chains from the TIL 5 TCR was cloned into a modified version of SAMEN vector. The IRES and neo^r sequences were removed from SAMEN during the construction of the A6 virus. Two transcripts were made from the A6 virus. One transcript was initiated from the promoter in the 5′ LTR and encoded both the α and β chains. The second transcript was initiated from the SR promoter and encoded only the β chain cDNA.*

stimulators in RPMI-10 without PMA. The amount of GM-CSF released in culture supernatants was measured by GM-CSF ELISA (R&D Systems).

Construction of the A6 Retrovirus

The structure of the A6 retrovirus is illustrated in *Figure 3*. Cloning of full length cDNAs encoding the Vα and Vβ genes from TIL clone 5 into pCR II and pCDL vectors and the shuttle vector containing the SAMEN retroviral backbone (pg1 SAMEN) have been described elsewhere.[17,30] An Xho I – Bgl II fragment containing the SRα promoter and the Vβ7.3 gene from pCDL/Vβ7.3 was ligated into the Xho I and Bam HI sites of the pg1 SAMEN. In the resulting construct, pg1 SAM/Vβ7.3, the IRES and neomycin regions of pg1 SAMEN are replaced with SRα- Vβ7.3 regions of pCDL/Vβ7.3. Next, an Xho I fragment containing the Vα1 gene from pCR II/Vα1 was ligated into the Xho I site of pg1 SAM/Vβ7.3. Restriction endonuclease and DNA sequence analysis of the resulting construct, designated A6 was performed to insure correct orientation and position of the TCR genes in the retroviral backbone. All restriction endonucleases and T4 DNA ligase was purchased from Boehringer Mannheim (Indianapolis, IN). DNA sequencing was performed in a Perkin-Elmer 9600 thermocycler (Norwalk, CT) using a cycle sequencing dye terminator kit (PE – Applied Biosystems Inc., Foster City, CA). Sequencing reactions were analyzed using an ABI 310 DNA sequence analyzer (Applied Biosystems Inc.).

Retroviral Supernatant Production

Stable retroviral producer cell lines were produced by co-transfecting A6 plasmid DNA together with pcDNA III (source of the neo^r gene for G418 selection; Invitrogen) into the various packaging cell lines using a Mammalian Calcium Phosphate Transfection Kit (Stratagene, La Jolla, CA). Tranfectants were selected and maintained in RPMI-10 medium supplemented with 400 μg/ml G418. Bulk producer lines were cloned by limiting dilution and high titer clones (>10^6 pfu/ml) were selected based upon their ability to transfer the A6 genome to NIH3T3 cells (amphotropic virus) or HCT116 cells (PG13 virus). For the production of retroviral supernatant, 4 x 10^6 producer cells were allowed to adhere to 75 cm^2 tissue culture flasks in RPMI-10 medium overnight at 32ºC in a humidified incubator containing 5% CO_2. The medium was replaced and retroviral supernatants were collected 16 hours thereafter.

Retroviral Transduction

Established cell lines were transduced using retroviral supernatants which were prepared as described above. For suspension cells (Jurkat), 7x10^5 cells were re-suspended in 5 ml of retroviral supernatant containing 8 μg/ml polybrene and placed in a six-well plate (Falcon/Becton Dickinson, Franklin Lakes, NJ). For adherent cells (NIH3T3 or HCT116), 2x10^5 cells were allowed to adhere to six-well plates in RPMI-10 medium overnight. Cells were centrifuged at 1000g for 2 hours at 32ºC. The medium was replaced and the cells were allowed to recover overnight at 37°C in a humidified incubator containing 5% CO_2. For bone marrow reconstitution assays, donor bone marrow was isolated from the femurs of C3H X C56Bl/6 F_1 mice. Erythrocytes were removed by diluting cells 1:4 in ice cold 7% ammonium chloride solution (Stem Cell Technologies, Vancouver, BC, Canada) and incubating on ice for 10 minutes with gentle mixing. Bone marrow cells were cultured in stem cell medium which consisted of RPMI-10 medium supplemented with 100 ng/ml SCF and 50 ng/ml IL-6 for 3 days. Bone marrow was added to six well plates containing 2x10^5 irradiated PA317/A6 producer cells (100 Gy) in retroviral supernatant supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, and 8 μg/ml polybrene and centrifuged at 1000g for 2 hours at 32ºC. The cells were allowed to recover overnight at 37°C in a humidified incubator containing 5% CO_2. It was repeated for a total of three transductions, than the marrow was then harvested and cultured for 24 hours in stem cell medium prior to use in reconstitution assays.

Bone Marrow Reconstitution of Lethally Irradiated HLA-A2/K^b Transgenic Mice

Transduced bone marrow was collected and re-suspended in HBSS at 2x10^6 cells/ml. Recipient mice were lethally irradiated with 9 Gy whole body -irradiation. Mice were reconstituted with 0.5 ml (1x10^6 cells) transduced bone marrow by tail vein injection.

Nucleic Acid Isolation

0.5-0.75 ml of blood was collected from the tail vein of mice in eppendorf tubes containing 0.1 ml of heparin. Erythrocytes were removed by diluting cells 1:4 in ice cold

7% ammonium chloride solution (Stem Cell Technologies) and incubating on ice for 10 minutes with gentle mixing. Cells were washed once in HBSS, counted, and pelleted for nucleic acid extraction.[31] For isolation of high molecular weight genomic DNA, cells were resuspended at 5 x 10^6 cells/ml in PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, and 50 mM KCl, 0.1 mg/ml gelatin) containing 0.5% Tween 20 (Fisher Scientific, Fairlawn, NJ) and 100 µg/ml Proteinase K (Boehringer Mannheim) and incubated at 56ºC for 45 minutes. Proteinase K was inactivated by heating at 95ºC for 10 minutes. Total RNA was isolated from PBMC using RNeasy Total RNA isolation kits (Qiagen, Chatsworth, CA). RNA was then treated with RNAse free DNAse (Gibco/BRL) to remove any contaminating genomic DNA that might be present.

PCR

For PCR amplification of genomic DNA, 1 µl of extracted DNA (equivalent to 5000 cells) was used in each reaction. For PCR amplification of RNA, first strand cDNA was first prepared from 1 µg of total cellular RNA using First Strand cDNA Synthesis kits (Gibco/BRL). The amount of cDNA equivalent of 50 ng of total RNA was used in each reaction. All PCR reactions were carried out in a 50 µl total volume containing PCR buffer, 200 µM dNTP (Gibco/BRL), 400 µM of each primer, and 1 unit of Taq DNA polymerase (Gibco/BRL) in a Perkin Elmer 9600 thermocycler (Norwalk, CT). Reactions were amplified for 35 cycles under the following conditions: 92ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 60 seconds. Reactions were seperated on 1% agarose gels containing 50 ng/ml ethidium bromide (Gibco/BRL) and bands were visualized with UV light.

Immunofluorescence

The MHC class I antigen expression on the PBMC of reconstituted mice was determined by immunofluorescence using anti-H-2 mAb. 1x10^5 RBC depleted PBMC were stained with 1.0 µg anti-H-2K^k-PE and 1.0 µg anti-H-2K^b-FITC mAb (PharMingen, San Diego, CA). After staining, propidium iodide was added to distinguish live cells from dead cells. Relative log fluorescence of 1x10^4 live cells was measured using a FACScan 440 (Becton Dickinson, Mountain View, CA).

Results

We have previously shown that the Vα1/Vβ7 TCR from TIL 5 mediated HLA-A2 restricted recognition of MART-$1_{(27-35)}$.[30] In order to more efficiently transfer the TIL 5 TCR to alternate effectors, the A6 retrovirus was constructed using a modified SAMEN backbone *(Figure 3)*. Since this virus contained two promoters, two transcripts were made from this virus. One transcript was initiated from the 5' MMLV LTR which contained the entire retroviral genome including both the Vα1 and the Vβ7 chains of the TIL 5 TCR. The second transcript was initiated from the SRα promoter which contained only the Vβ7 chain of the TIL 5 TCR. A6 producer cell lines were made which produced virus containing the ecotropic envelope (GP&E 86, infects only murine cells), the amphotropic envelope (PA317, infects both murine and human cells), or the Gibbon ape envelope (PG13, infects only human cells). We found that the PA317-A6 virus was more efficient in transducing NIH3T3 cells and murine bone marrow cells than the GP&E 86-A6 virus and the PG13-A6 virus was more efficient in transducing HCT116 cells cells and G-CSF mobilized $CD34^+$ human peripheral blood progenitors than the PA317-A6 virus (data not shown). Therefore, the PA317-A6 virus was used for all subsequent murine cells transductions and the PG13-A6 virus was used for all subsequent human cell transductions.

The ability of the A6 virus to transfer functional TIL 5 TCR to alternate effectors was tested by transducing Jurkat cells and measuring their ability to recognize MART-$1_{(27-35)}$ peptide. As shown in *table 4,* Jurkat cells transduced with the PG13-A6 virus secreted IL-2 when stimulated with T2

Table 4. **MART-$1_{(27-35)}$ Recognition by TIL 5 TCR Transduced Jurkat Cells**

	Responder Cells[1]					
Stimulator	*Untransduced Jurkat*	*Clone 22 Jurkat*	*PG13 Bulk Transduced Jurkat*	*PG13 Clone 1 Transduced Jurkat*	*PG13 Clone 2 Transduced Jurkat*	*PG13 Clone 4 Transduced Jurkat*
none	0.2	0.0	0.0	0.0	0.5	0.0
OKT3	433.3	765.6	1135.5	611.6	379.2	312.1
T2, unpulsed	0.0	0.0	0.0	0.0	0.0	0.5
T2 + MART-$1_{(27-35)}$	1.5	1210.6	3.2	21.8	38.1	1.1
T2 + $gp100_{(209-217)}$	0.0	0.4	0.3	0.3	0.0	3.1

[1]pg/ml IL-2 released

cells pulsed with MART-$1_{(27-35)}$ peptide but not unpulsed T2 cells or T2 cells pulsed with $gp100_{(209-217)}$ peptide. In contrast, untransduced Jurkat cells failed to recognize MART-$1_{(27-35)}$ peptide pulsed T2 cells. Although the absolute amount of IL-2 produced by the transduced Jurkat cells was comparatively low relative to clone 22 (38.1 pg/ml vs 1210.6 pg/ml), a Jurkat clone stably transfected with the TIL 5 TCR, the pattern of recognition indicated the expression of a functional TIL 5 TCR on the Jurkat transductants. Therefore, the A6 retrovirus can transfer the reactivity and specificity of TIL 5 to alternate effector cells.

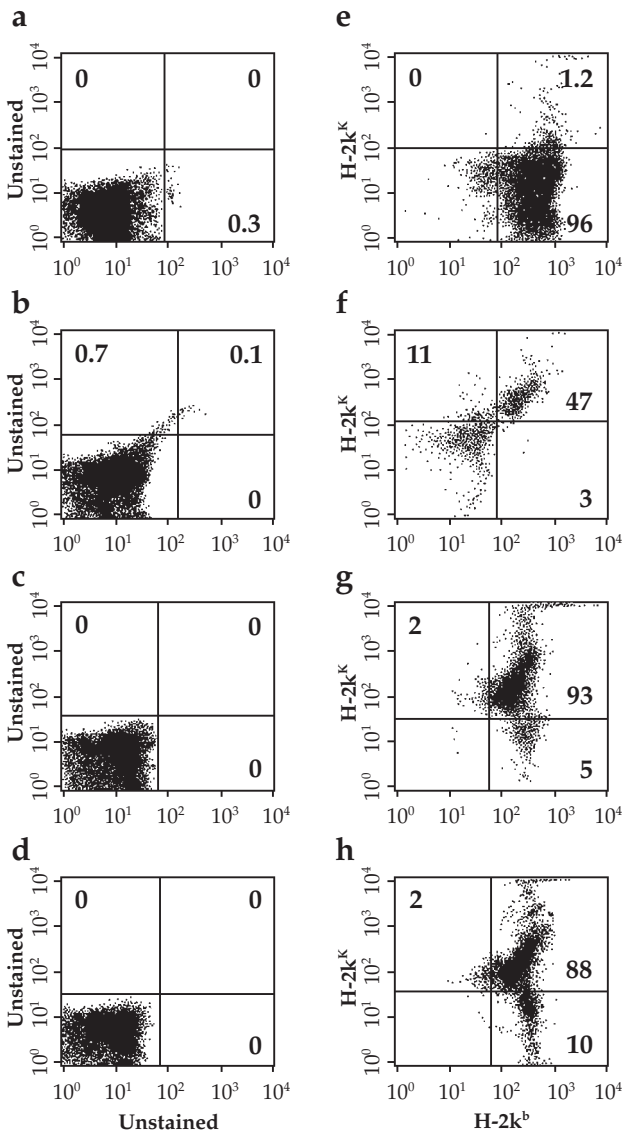

Figure 4. *Immunofluorescence analysis of PBMC from bone marrow reconstituted lethally irradiated mice. PBMC from two representative bone marrow reconstituted mice (panels c,d,g,h), one representative recipient HLA-A2 transgenic mouse (panels a,e), and one representative donor C3H x C57Bl/6 F_1 mouse (panels b,f) were stained with FITC conjugated H-2K^b and PE conjugated H-2K^k mAbs to monitor their MHC class I antigen expression. The fluorescence profiles from unstained PBMC (panels a-d) were compared to stained PBMC (panels e-f) to determine the percent donor and recipient PBMC in each group. Each histogram represents the relative log green and red fluorescence of at least 6,500 live cells.*

An animal model was established to ascertain the feasiblity of transferring human TCR genes to alternate effectors by transducing hematopoietic stem cells. Bone marrow from C57Bl/6 x C3H F_1 mice were transduced with the PA317-A6 virus and used to engraft lethally irradiated HLA-A2/K^b transgenic mice. Four weeks post-engraftment, PBMC from engrafted mice were screened by immunofluorescence for evidence of reconstitution. PBMC from recipient mice prior to bone marrow transfer expressed only H-2K^b *(Figure 4*, panel e) whereas PBMC from donor mice expressed both H-2K^b and H-2K^k *(Figure 4,* panel f). Greater than 90% of the PBMC from two representative mice engrafted with transduced bone marrow expressed H-2^k indicating that the mice were successfully reconstituted *(Figure 4* panels g and h). The results shown are typical of engrafted mice in our experiments with each mouse having a minimum of 80% donor derived PBMC and most animals having >90% donor derived PBMC. Reconstituted mice survived up to a year post-engraftment and maintained high levels (>85%) of donor derived PBMC (data not shown). Therefore, we were able to successfully reconstitute lethally irradiated mice with transduced and untransduced bone marrow and cells derived from the donor bone marrow could be distinguished from host cells by their expression of H-2^k. Mice reconstituted with TIL 5 TCR transduced bone marrow were screened for the presence of the transgenes by PCR using human TCR specific primers. High molecular weight DNA was prepared from the PBMC of these mice as early as 4 weeks post-engraftment and at monthly intervals through week 40. We were able to amplify a 200 bp band corresponding to the TIL 5 TCRα C region from the PBMC of mice engrafted with transduced bone marrow as early as 4 weeks (data not shown) and as late as 40 weeks after reconstitution *(Figure 5 a,* lanes 3–10). We were unable to detect TCRα chain bands from the PBMC of control mice reconstituted with untransduced bone marrow *(Figure 5 a,* lanes 1,2). Similarly, a 500 bp band was amplified using primers specific for the TIL 5 TCRβ chain *(Figure 5 b*, lanes 3–10). These results indicate the A6 retrovirus can transfer the TIL 5 TCR to cells capable of reconstituting a lethally irradiated animal and the provirus was maintained long after engraftment.

Expression of the TIL 5 TCR in the PBMC of mice reconstituted with gene modified bone marrow was measured by RT-PCR using TIL 5 TCR α and β chain specific primers. As shown in *Figure 6*, both TIL 5 TCR α and β chain transcripts were detected 40 weeks after engraftment with transduced bone marrow (panels a and b, lanes 3–10). TIL 5 TCR transcripts were not detected in control mice

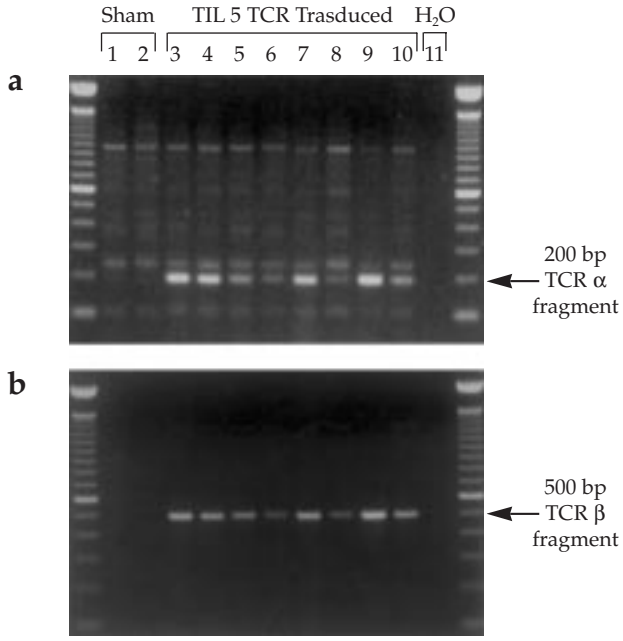

Figure 5. *TIL 5 TCR α and β genes in the PBMC of bone marrow reconstituted mice. The presence of the A6 provirus in the PBMC of bone marrow reconstituted mice was determined by PCR using primers which amplify the TIL 5 TCR α and β chains. Primers CαCON F and CαCON R amplified a 200 bp band corresponding to the Cα region of the TIL 5 TCR α chain from the A6 virus (panel a). Primers TIL 5β VDJ and CβCON R amplified a 500 bp band corresponding to the TIL 5 TCR β chain from the A6 virus (panel b). High molecular weight genomic DNA was prepared from the PBMC of mice engrafted with sham transduced (lanes 1 and 2) and A6 transduced (lanes 3–10) mice. "No template" reactions were performed to control for artifacts resulting from PCR contamination (lane 11).*

engrafted with sham transduced bone marrow *(Figure 6,* panels a and b, lanes 1 and 2). No bands were seen in the -RT control reactions confirming that the observed bands were not due to contaminating genomic DNA in the RNA preparations *(Figure 6,* panels c and d). The presence of transcripts encoding TIL 5 TCR α and β chains in the PBMC of mice more than 6 months after reconstitution suggests that long term expression of the TIL 5 TCR can be transferred to alternate effectors using the A6 retrovirus.

The persistence of TIL 5 TCR α/β chain DNA and RNA in the PBMC of mice more than six months after reconstitution is suggestive but is not definitive proof that the mice were sucessfully reconstituted with transduced stem cells or lymphoid progenitors. In order to confirm that true stem cells or lymphoid progenitors were transduced, bone marrow was harvested from a mice engrafted with TIL 5 TCR transduced or untransduced bone marrow. This bone marrow was used to reconstitute a second group of lethally irradiated HLA-A2 transgenic mice. TIL 5 TCR α and β chain DNA and RNA was detected in the PBMC of the mice which received bone marrow from the TIL 5 TCR α/β chain$^+$ mouse but not the TIL 5 TCR α/β chain$^-$ mouse (data not shown). Detection of TIL 5 TCR α/β chain DNA and RNA in the PBMC of a second group of mice which were reconstituted with bone marrow from a reconstituted mouse confirms that we successfully transduced true stem cells or lymphoid progenitors.

Discussion

We have constructed a retrovirus containing the TCR α and β chains of an HLA-A2 restricted, MART-1 reactive CTL clone (TIL 5). This TCR mediates recognition of MART-1 peptide in a CD8 co-receptor-independent manner, and is probably a high affinity TCR.[30] The virus can transfer the reactivity and specificity of this CTL clone to Jurkat cells. Using this retrovirus, we were successful in transducing murine bone marrow derived hematopoietic stem cells. Lethally irradiated HLA-A2 transgenic mice engrafted with the gene modified bone marrow were fully reconstituted within 4 to 6 weeks. The TCR α and β transgenes could be detected in the peripheral blood of the reconstituted mice as early as 4 weeks post-engraftment by PCR. The transgenes were maintained in the peripheral blood of the engrafted mice for at least 40 weeks suggesting that true hematopoietic stem cells were transduced. TIL 5 TCR α and β transcripts could be detected by RT-

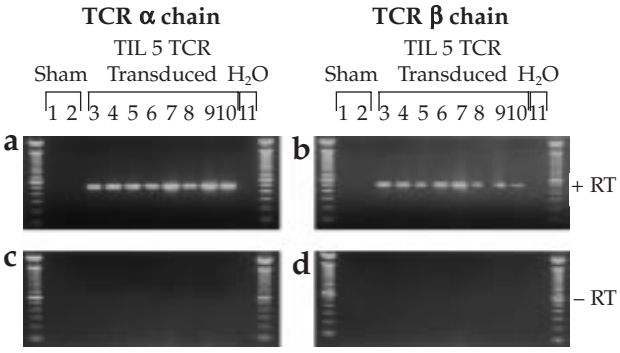

Figure 6. *TIL 5 TCR α and β transcripts in the PBMC of bone marrow reconstituted mice. The presence of the A6 transcripts in the PBMC of bone marrow reconstituted mice was determined by RT-PCR using primers which amplify the TIL 5 TCR α and β chains. Primers TIL 5α VJ and CαCON R amplified a 500 bp band corresponding to the TIL 5 TCR α chain from the A6 virus (panel a). Primers TIL 5β VDJ and Cβ CON R amplified a 500 bp band corresponding to the TIL 5 TCR β chain from the A6 virus (panel b). cDNA was prepared from total RNA isolated from the PBMC of mice engrafted with sham transduced (lanes 1 and 2) and A6 transduced (lanes 3–10) mice. "No template" reactions were performed to control for artifacts resulting from PCR contamination (lane 11). "No reverse transcriptase" reactions were performed to control for the presence of genomic DNA in the RNA samples (panels c and d).*

Table 5. **Recognition of Murine MART-1 by HLA-A2 Restricted Human TIL**

		Responder Cells[1]	
Cell Line	*Transfected Gene(s)*	*TIL1200*[2]	*TIL1235*[3]
none	none	4.9	16.0
COS-7	HLA-A2	4.2	20.5
COS-7	HLA-A2 + h-gp100	149.4	24.0
COS-7	HLA-A2 + h-MART-1[4]	9.0	1019.0
COS-7	HLA-A2 + m-MART-1[5]	9.4	2003.0

[1]pg/ml GM-CSF released
[2]TIL 1200 recognized gp100 in the context of HLA-A2.
[3]TIL 1235 recognized MART-1 in the context of HLA-A2.
[4]Human MART-1.
[5]Murine MART-1.

PCR indicating that the transgenes were expressed in the PBMC of the bone marrow reconstituted animals. The sustained expression of TIL5 TCR α and β transcripts in secondary recipients, following the transfer of bone marrow from a mouse previously reconstituted with transduced bone marrow, confirms that a true pluripotent stem cell or lymphoid progenitor cell was transduced.

Currently, we have no evidence that mouse T cells in the periphery of reconstituted mice are expressing the human TIL 5 TCR. In a recent study, peripheral T cells of mice reconstituted with bone marrow transduced with a murine ovalbumin ($OVA_{257-264}$)-specific / TCR did express the introduced TCR on the cell surface.[10] This demonstrated that expression of the $OVA_{257-264}$-specific TCR was sufficient to permit T cell progenitors to differentiate in the thymus. However, Pogulis et al. did not show antigen-specific recognition by these T cells. This could be due to a defect in the cloned α and/or β chain sequences of the TCR, since Pogulis et al. have also not previously demonstrated that this TCR could mediate recognition of $OVA_{257-264}$ peptide using, for example, transfected Jurkat cells or transduced peripheral blood T lymphocytes. The TIL 5 TCR has previously been shown to function in Jurkat transfectants[30] and retrovirally transduced human peripheral blood T lymphocytes (Clay et al., submitted). Additionally, we have now shown that transduction with the A6/PA317 virus can transfer TIL 5 TCR function to Jurkat cells. we expect the TIL 5 TCR to mediate antigen-specific recognition in transduced mouse T cells that express this TCR. However, one caveat of using the TIL 5 TCR is that the human MART-1 epitope recognized by this TCR has a single amino acid substitution in the equivalent epitope of mouse MART-1. This could prevent the survival of mouse T cells expressing the TIL 5 TCR during thymic selection, since these T cells may not be "positively selected" and/or may be "negatively selected". For this reason we are currently cloning TCRs that recognize TAA epitopes that are conserved in mouse and human. However, it should be noted that TIL 1235, a MART-1 reactive human TIL culture, did recognize COS cells transfected with HLA-A2 and the mouse or human MART-1 gene *(Table 5),* so it is possible that TIL 5 TCR transduced mouse T cells will also recognize the mouse MART-1 epitope and survive thymic selection.

This study represents a preclinical animal model for the transfer of the genes encoding a melanoma reactive TCR to alternate effectors. By genetically modifying hematopoietic stem cells with the TCR α and β chains from a tumor reactive CTL clone, the presence of the productively rearranged TCR in T cell progenitors during T cell development should prevent rearrangement of the endogenous TCR genes by allelic exclusion. Therefore, T cells derived from the transduced stem cells should only express the introduced TCR and thus possess the specificity and reactivity of the original T cell clone. The transfer of tumor reactive TCR genes into hematopoietic stem cells has therapeutic potential. Engrafting patients with genetically modified hematopoietic stem cells should increase the overall frequency of tumor reactive T cells bearing a known TCR in their peripheral blood. Also, the frequency of T cells bearing the introduced TCR should be relatively high. Furthermore, if a true pluripotent stem cell is transduced, the self-renewing capacity of this cell should result in sustained levels of T cells expressing the tumor reactive TCR.

Several critical questions can be addressed by expressing tumor reactive TCR genes in hematopoietic stem cells. Firstly, we can evaluate the effectiveness of targeting individual peptide epitopes for immune therapy. The ability to transfer the reactivity and specificity of T cell clones to alternate effectors will enable us to treat different patients with essentially the same T cell clone. This was not possible in clinical trials using the adoptive transfer of TIL because of the oligoclonal nature of most TIL cultures. TCR gene transfer will also enable treatment strategies to focus on epitopes that are otherwise poorly immunogenic when patients are vaccinated with peptide or recombinant viruses, provided that a suitable T cell clone is available for isolation of the α/β TCR genes. These studies should permit the identification of the most effective epitopes for patient treatment. Secondly, transferred TCR genes can provide marker genes for monitoring purposes. In order to gain insights into the T cell mediated immune response against tumors *in vivo*, we want to study how long adoptively transferred cells remain in the periphery of patients and if these cells traffic to tumor deposits. The unique CDR3 sequences of the TCR α and β chains will permit the levels of TCR gene modified T cells in the periphery of patients to be determined using competitive PCR (McKee et al., in press). Additionally, PCR of fine-needle

aspirates from tumor lesions will allow trafficking of gene modified TCR-expressing T cells to tumor to be assessed. The trafficking of TCR transduced T cells to regressing versus non-regressing lesions will be compared.

Unfortunately, no gene therapy trial to date has been conclusively proven to be effective in treating the targeted disease. As a result, a panel of experts was critical of most gene therapy trials in their report to the Director of the National Institutes of Health.[32] It is clear that greater emphasis should be placed in vector development and understanding the biology of gene therapy targets if we expect gene therapy to be a viable treatment option in the future. The use of animal models such as this should provide a greater understanding of the obstacles to be overcome if the clinical potential of gene therapy is to be realized. Further advances will also be required in vector development and in establishing the optimum transduction conditions for target cells to enhance the efficiency of gene transfer and to provide prolonged gene expression.

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